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Physiological, structural, and immunological characterization of leaf and chloroplast development in cotton

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Summary. Many of the studies of chloroplast ontogeny in higher plants have utilized suboptimal conditions of light and growth to assess development. In this study, we utilized structural, immunological, and physiological techniques to examine the development of the chloroplast in fieldgrown cotton (*Gossypium hirsutum* cv. "MD 51 ne"). Our youngest leaf sample developmentally was completely folded upon itself and about 0.5 cm in length; leaves of this same plastochron were followed for three weeks to the fully expanded leaf. The chloroplasts at the earliest stage monitored had almost all of the lamellae in small, relatively electron-opaque grana, with relatively few thylakoids which were not appressed on at least one surface. During the development of the thylakoids, the membranes increase in complexity, with considerable stroma lamellae development and an increase in the number of thylakoids per granum. Besides the increase in complexity, both the size and numbers of the chloroplast increase during the development of the leaf. Developmental changes in six thylakoid proteins, five stromal proteins, and one peroxisomal protein were monitored by quantitative immunocytochemistry. Even at the earliest stages of development, the plastids are equipped with the proteins required to carry out both light and dark reactions of photosynthesis. Several of the proteins follow three phases of accumulation: a relatively high density at early stages, a linear increase to keep step with chloroplast growth, and a final accumulation in the mature chloroplast. Photosystem-II(PS II)-related proteins are present at their highest densities early in development, with an accumulation of other parts of the photosynthetic apparatus at a latter stage. The early accumulation of PS-II-related proteins correlates with the much lower ratio of chlorophyll *a* to *b* in the younger leaves and with the changes in fluorescence transients. These data indicate that some of the conclusions on chloroplast development based upon studies of intercalary meristems of monocots or the greening of etiolated plants may not be adequate to explain development of chloroplasts in leaves from apical meristems grown under natural conditions.

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Abbreviations: CF1 chloroplast coupling factor 1; chl chlorophyll; DAP days after planting; LHC light-harvesting chlorophyll-*a*/*b*-binding protein; OEC oxygen-evolving complex of photosystem II; PBS phosphate-buffered saline; PS photosystem; RuBisCo ribulose bisphosphate carboxylase/oxygenase.

Introduction

Much of what we know about the sequence of chloroplast development in higher plants has come from studies of the greening of etiolated plants after exposure to light or the changes from the base to the tip of monocot leaves derived from intercalary meristems (Leech 1984, Whatley 1978, Mayfield and Taylor 1984). Both of these systems allow for good reproducibility of results and may be followed with biochemical studies to support their findings. Leaves of dicotyledons and many nongrass monocotyledons do not develop from intercalary meristems, however, nor are most plants exposed to the conditions of uninterrupted darkness prior to leaf development. Thus, the model of chloroplast development of land plants is based upon systems that may or may not be typical of land plants with an apical meristem. Moreover, most of these studies have relied upon growth conditions that would certainly not have maximized photosynthesis, such as growing plants in growth chambers or in dimly lighted greenhouses during the winter months (Leech 1984). These growth conditions would alter both the spectra and quanta of light per-

ceived by the plant, both of which could seriously alter patterns of leaf and chloroplast development (Smith and Longstreth 1994, Mohr 1984). Therefore, there is still a great deal of uncertainty as to how chloroplasts develop in leaves from the apical meristems of dicotyledonous plants and under natural conditions of light and growth.

The diverse literature on the development of chloroplasts in angiosperms has been summarized by several reviewers (Whatley 1974, 1978; Whatley et al. 1982; Wellburn 1984) and a basic model of chloroplast development has been devised (Whatley 1978), based upon the monocot intercalary meristem and what little work has been attempted on development from apical meristems of dicots. At the meristem, eoplasts or proplastids with little internal membranes are found. These develop into starch-filled amyloplasts, from which develop plastids with perforated lamellae and prothylakoid bodies found in etioplasts. Eventually the thylakoids begin to overlap, creating small grana membrane stacks. Further chloroplast development results in larger, more extensively stacked, mature chloroplasts. Studies of intercalary meristems of monocots (e.g., Baker et al. 1983), cytochemical studies on developing plastids (Wrischer 1988, 1989), and combined structural and immunofluorescence studies (Robertson et al. 1993, Robertson and Leech 1995) have attempted to correlate structure and function in chloroplast development.

We have begun a series of structural and physiological investigations of leaf and chloroplast development in field-grown cotton plants (Pettigrew et al. 1993). The data presented in this study follow the development of a leaf at a given plastochron from a tightly folded to a fully expanded leaf. A separate report will concentrate on chloroplast development from the apical plastids to the earliest stage monitored herein which were so small that physiological measurements could not be obtained. For each of the sampling dates, we examined the chlorophyll content, fluorescence kinetics, structure, and presence and amount of chloroplast proteins by immunogold electron microscopy. Antisera to twelve different proteins (six thylakoid, five stromal, and one peroxisomal) were used to give a rather complete perspective of the changes in chloroplast components over development.

Material and methods

Plant material

Field plots of the cotton (*Gossypium hirsutum* L., genotype "MD 51 ne") were established on a Bosket fine sandy loam (fine-loamy, mixed, thermic mollic Hapluddalfs) near Stoneville, MS. The experimental area was planted 22 April 1994. A randomized complete block experimental design with six replicates was utilized. Unexpanded leaves, approximately 0.5 cm in length from a large number of plants, all of the same plastochron, were tagged at 52 days after planting (DAP). These leaves were designated "day 0", with subsequent collections 3, 8, 11, and 21 days after tagging.

Fluorescence induction kinetics

Chlorophyll (chl) fluorescence was measured on two of the tagged leaves per plot at 3, 8, 11, and 21 days after tagging with a CF-1000 chl fluorescence measurement system (P.K. Morgan, Inc., Andover, MA). Leaves were allowed to dark adapt for at least 30 min prior to measurements, and all measurements were completed at least 2 h before solar noon.

Leaf area

Following the chl fluorescence determinations, one of the two leaves per plot, six total, were harvested and used for leaf area measurements with a CI-203 laser area meter (CID Inc., Vancouver, WA).

Chlorophyll concentration determination

After leaf area determinations were made, the leaf was divided in half, with one half used for microscopy, the other for chl determination. Leaf discs were punched and chlorophyll extracted in 95% ethanol at 30 °C in the dark. Chl was quantified by the equations of Holden (1976).

Electron microscopy

Leaf sections were minced into 1 mm^2 pieces in a drop of 6% (v/v) glutaraldehyde in 0.05 M Pipes buffer (pH 7.4) and then fixed for 2 h at room temperature in a vial of this same fixative. The samples were washed twice in 0.10 M cacodylate (pH 7.2) for 15 min, and post-fixed in 2% (w/v) osmium tetroxide in cacodylate buffer for 2 h. After a brief rinse in distilled water, the samples were then stained en bloc in 2% (w/v) uranyl acetate for 18 h at 4 °C. After several distilled-water rinses, the samples were dehydrated in a graded acetone series, transferred to propylene oxide, and then embedded in a 1 : 1 mixture of Spurr's low viscosity and Polybed 812 resins (Polysciences Inc., Warrington, PA). Samples were flat embedded in Beem embedding molds and remounted on epoxy stubs to obtain cross-sections. Sections were cut with a diamond knife on a Reichert Ultracut ultramicrotome with pale-gold-to-silver reflectance. Sections were post-stained with 2% (w/v) uranyl acetate and Reynolds's lead citrate for 7 min each before observation.

Immunocytochemistry

Sections from the same leaf pieces used for electron microscopic analysis were fixed in 3% (v/v) glutaraldehyde in 0.05 M Pipes buffer (pH 7.4) for 2 h at 4 °C, washed in several exchanges of cold buffer, and then dehydrated in ethanol. The first dehydration steps to 75 % (v/v) were carried out at 4 °C, with subsequent processing steps carried out at –20 °C. Material was gradually infiltrated with LR White resin medium grade, with a minimum of 1 day per each 25% increase in concentration. Blocks were polymerized either by the LR White chemical catalyst (0.10 ml catalyst per 20 ml of resin) in Beem capsules at –20 °C or by heat at 60 °C for 1 h. These two methods were used for each tissue sample to obtain maximal labelling for a specific antibody/antigen combination.

Sections with pale-gold-to-silver reflectance colors were cut with a Reichert Ultracut ultramicrotome and mounted on either Formvar-coated or uncoated gold grids (300 mesh). The specimens were then floated on 4 µl drops of the following: 1% (w/v) non-fat dry milk in phoshate-buffered saline (PBS), 30 min; primary antibody diluted in 1% (w/v) BSA in PBS, 4 h [The concentrations of primary antibody employed were determined empirically. Concentrations of primary antibody that gave background reaction on areas of plastic or cell wall at or below 0.3 gold particles per μm^2 were utilized. These were generally about 10-fold more concentrated than that dilution of primary antibody that gave a strong reaction on Western blots. All of the antibodies used give single reactive polypeptide bands on Western blots of SDS-solubilized chloroplast preparations, with the exception of the CF1 serum, which recognizes the alpha and beta subunits in the complex. Sources of many of these antisera are described in Lax and Vaughn (1991); others were produced by commercial antisera laboratories with purified proteins prepared in our laboratory as antigens.]; PBS-BSA, four times, 2.5 min each; 1 : 20 dilution of Protein A colloidal gold or goat anti-mouse IgG colloidal gold (EY Labs, San Mateo, CA); PBS, four times, 2.5 min each. After washing thoroughly with spray of distilled water, the grids were post-stained in 2% (w/v) uranyl acetate (4 min) and Reynolds's lead citrate (30 s) before observation.

Microscopic sampling and statistics

At each collection date, six samples were obtained, with half of each sample randomly utilized for standard microscopy or immunocytochemistry. Chloroplast volumes were calculated utilizing the formula for the volume of an ellipse, with chloroplast depth estimated as half of the chloroplast width. For morphometric analysis of electron microscopic specimens, all of the plastid profiles within a grid square of a 300-mesh grid nearest to the center point of observation in the Zeiss EM 10 CR were photographed, so as to assure that they were randomly selected. Counts of colloidal gold particles were obtained from similarly randomly selected plastids, except that only full plastid profiles were utilized. Chloroplast and peroxisome areas and gold densities were determined as described previously (Vaughn 1986, 1989; Vaughn et al. 1990). For the immunogold localizations, each antiserum was utilized in a minimum of three replicates, utilizing aliquots of the same antiserum dilution and gold solutions, to assure reproducibility. Data from all of these studies were subjected to analysis of variance. Means were separated by use of a projected LSD ($P < 0.05$).

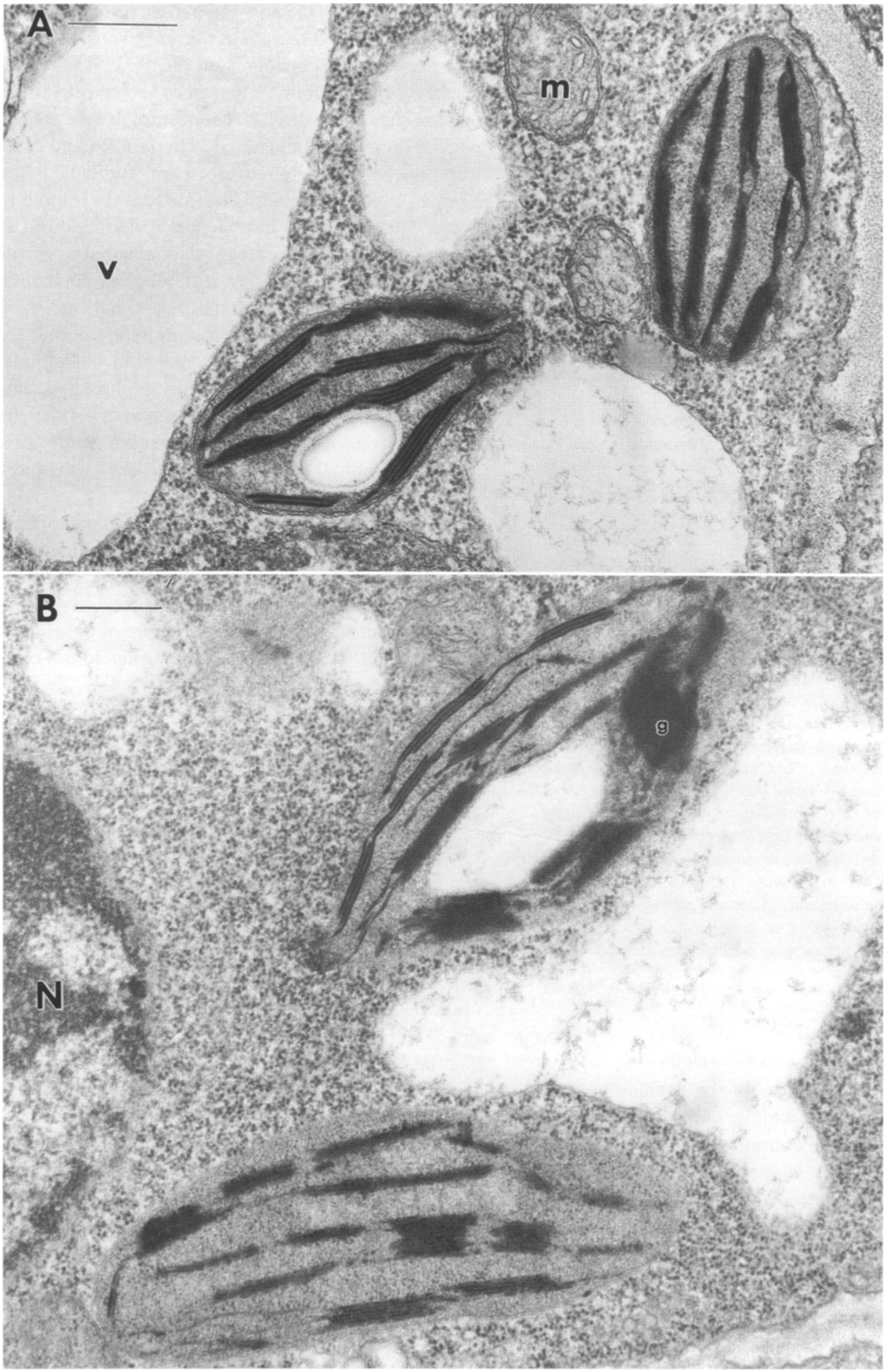
Results

Ultrastructure of chloroplast development

At 0 days, the chloroplasts of the developing cotton leaf (Fig. 1 A) are strikingly unlike those reported previously for mature cotton leaves (Lax et al. 1987, Bondada et al. 1994). Chloroplasts are ca. 1.5 µm in length and are dispersed throughout the cytoplasm, with no close organellar association, with the exception of mitochondria. Thylakoids are relatively more electron opaque compared to the envelope (and vesicles apparently derived from it) and stroma. The individual grana do not contain many thylakoids but all of the thylakoids are appressed virtually all along their entire length, with the only unappressed areas consisting of the tops and bottom of the stack and very short (less than 0.1 µm) unappressed membranes that connect the adjacent grana. Ribosomes and fibrillar areas that are sites of plastid DNA concentration are prominent in these chloroplasts. Starch granules and electron-opaque plastoglobuli are found throughout the stroma. Both chloroplast and nuclear divisions are noted. Other workers have reported that chloroplast development is complicated in dicotyledonous plants as the tissue is a mosaic of different developmental stages within the leaf. However, we found that, even at this young stage, the six different plant samples are amazingly consistent. Moreover, within the sample, all of the tissue layers had similar morphology at this developmental stage with the exception of cells in the paraveinal mesophyll cells. Chloroplasts in the paraveinal mesophyll cells are distinguished by the presence of electron-opaque inclusions and reduced development all through leaf development. These chloroplasts are not included in any of the quantification studies in this experiment.

After three days of development, there have been prominent changes in the ultrastructure of the chloroplasts (Fig. 1 B). The thylakoids are much less electron opaque than in the day-0 chloroplasts and the thylakoids are arranged into a more normal architecture with grana stacks around 0.5 µm in length and clear regions of unstacked stroma lamellae connecting the stacks. The plastids have expanded in both length and in volume (Table 1). As in the day-0 samples, only associations between chloroplast and mitochondria are noted and the chloroplasts appear to be randomly distributed through the cytoplasm. Division figures of both nucleus and chloroplast are noted. Grana stacks have increased in numbers of thylakoid per stack, despite the organization of thylakoids into areas of unstacked stroma lamellae compared to the day-0 chloroplasts.

The latter stages of leaf development are characterized by the elaboration of the thylakoid system and the expansion of the size of the plastid. Samples collected on days 8 and 11 are very similar in morphology and are considered here together. The chloroplasts are ca. 6 µm in length and contain well developed grana stacks and clearly differentiated stroma lamellae (Fig. 2 A). Virtually no chloroplast or nuclear divisions are observed at this stage of development. The cells in these stages are much more vacuolated, so that all the organelles are concentrated in the cell periphery. Peroxisomes are now prominent and are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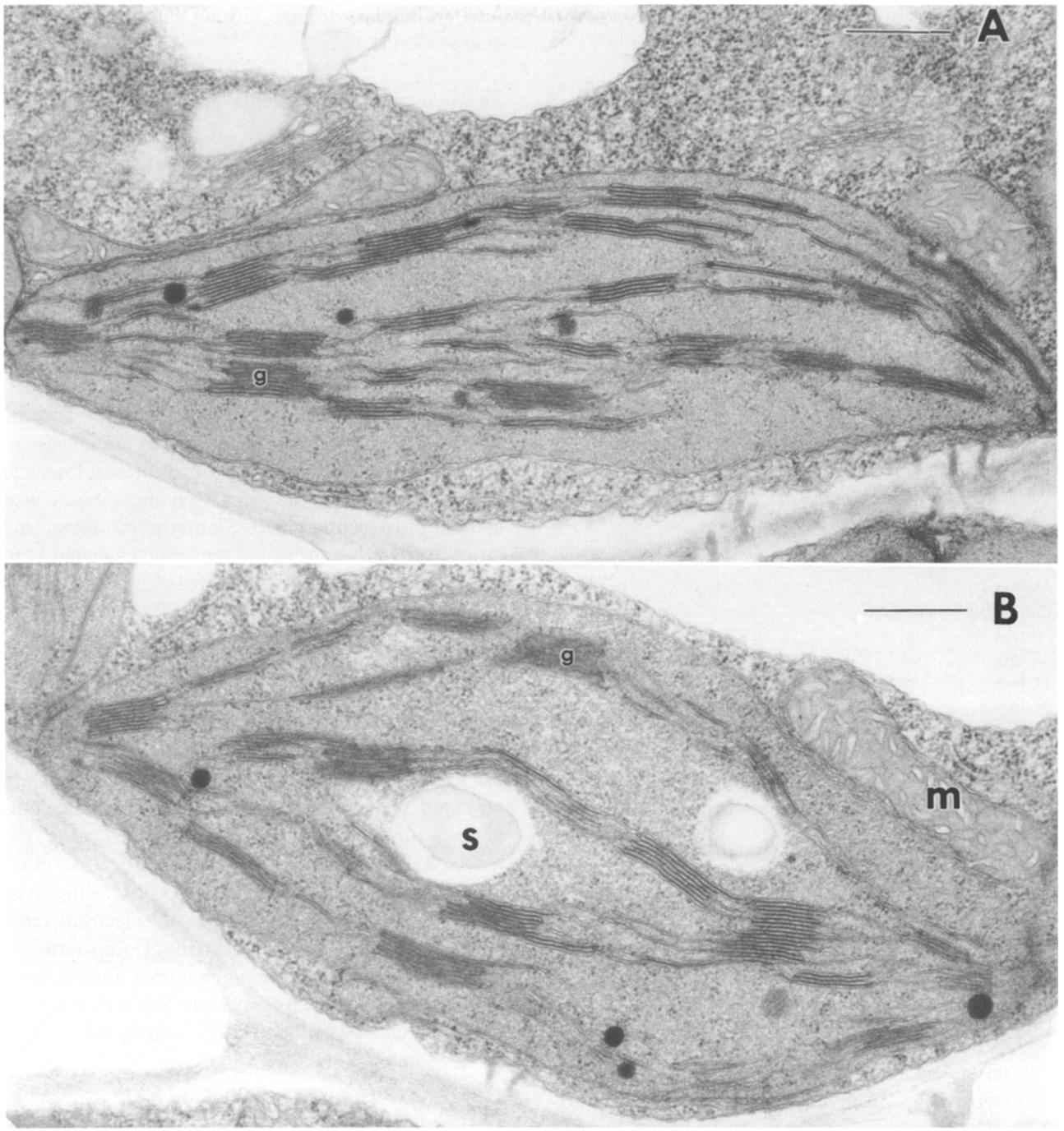

Fig. 2. Electron micrographs of chloroplasts from 11- (**A**) and 21-day (**B**) cotton leaves. These chloroplasts exhibit the ultrastructure typical of C3 chloroplasts, with thylakoids organized into grana stacks (*g*), interconnected by unappressed lamellae and a stroma containing abundant ribosomes and starch (*s*). Compare these much less electron-opaque thylakoids with those in the immature chloroplasts in Fig. 1. *m* Mitochondrion; bars: 0.5 μm

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Fig. 1 A, B. Electron micrographs of chloroplasts from 0- and 3-day cotton leaves. **A** Chloroplasts at 0 day contain electron-opaque thylakoids that occur almost completely in the appresssed form, with few areas in which the grana are interconnected by short distances of unappressed lamellae. **B** At 3 days, the thylakoids are electron opaque, but less so than at 0 day. In addition, the chloroplasts have increased in size and the thylakoids are organized into small grana, with more extensive unappressed lamellae connecting the stacks. *g* Granum, *m* mitochondrion; *v* vacuole; *N* nucleus; bars: 1.0 μm

Table 1. Morphometric measurements of chloroplast components in developing cotton leaves

Component/leaf age	% volume	Grouping[a]
Grana		
0 days	44.98	B
3 days	25.22	A
11 days	38.4	A
21 days	38.94	A
Stroma lamellae		
0 days	2.92	C
3 days	13.34	B
11 days	18.70	A
21 days	20.18	A
Stroma		
0 days	43.96	A
3 days	45.33	A
11 days	27.20	B
21 days	37.60	B
Inclusions[b]		
0 days	8.11	B
3 days	16.09	A
11 days	15.94	A
21 days	9.26	B
Chloroplast volume (μm^3)		
0 days	0.827	C
3 days	2.083	C
11 days	29.96	B
21 days	45.65	A

[a]Groups established statistically as being significantly different from those with another letter designation
[b]Starch, plastoglobuli

appressed to the chloroplast envelope and are generally found in a three-organelle association of peroxisome, chloroplast, and mitochondrion (not shown here but readily observed in the glycolate oxidase immunogold localizations below).

The samples collected 21 days after tagging contained chloroplasts that are typical of C3 chloroplasts of higher plants. The thylakoids are well-differentiated into unstacked and stacked grana regions, with stacks as extensive as 22 thylakoids in one granum (Fig. 2 B). These chloroplasts have the largest volume of any of the samples and contain large starch grains as well as plastoglobuli. Peroxisomes, prominent and greatly expanded over previous stages, are associated closely with the chloroplast.

Morphometric analysis of plastid profiles (Table 1) allows one to quantify the trends from observation of micrographs. Dramatic shifts in the percentages of chloroplast volume occupied by grana and stroma lamellae are obvious, although changes in the relative percentages of the stroma or stromal inclusions in the total chloroplast volume did not show any dramatic change (Table 1).

Physiological measurements

The quantity of chl on a leaf area basis increased approximately 4-fold from 0 to 21 days after tagging, but the amounts of the two chls change differently (Table 2). Chl-*a* levels increase 4-fold as do total levels, whereas chl-*b* levels change only twofold during the 21 days of development. The 0-day leaves have a ratio of 1.92 of chl *a* to *b* compared to the 3.65 at 21 days, a value typical of C3 dicots. Chl-*a* levels increase more rapidly than chl *b* at all times with the exception of the day-11 to the day-21 levels, in which there appears to be a greater increase in chl *b*. The low ratios of chl *a* to *b* in the day-0 leaves are consistent with the completely appressed membranes found in the chloroplasts as the appressed membranes also contain most of the chl *b*. Similarly, the increasing ratios of chl *a* to *b* during the transition from day 11 to 21 are consistent with the increase in unappressed membrane in these samples (Table 2).

The fluorescence induction curves also exhibited dramatic changes during the course of the development of the cotton leaf (Table 3). In the 3-day leaves, the parts of the induction curve associated with the primary photochemistry of PS II (the I and D portions of the curve, Briantais et al. 1986), are essentially obscured, and it is not until 8 days that the three components of the fluorescence induction curve are resolved (not shown). In later stages of leaf development, the quenching of the slow portion of the fluorescence curve indicates meaningful electron transport and CO_2 fixation are occurring. Lower ratios of the variable fluorescence to maximal fluorescence (F_v/F_m) in younger leaves indicate less efficiency for PS-II-associated photochemistry than in the 11- and 21-day samples (Table 3).

Table 2. Chlorophyll measurements and ratios of chl *a* to *b* for developing cotton leaves

Leaf age[a]	Concentration (mg/cm^2)	Ratio
0 days	176	1.92
3 days	172	2.39
8 days	314	3.19
11 days	339	3.96
21 days	542	3.65
LSD 0.05	41	0.41

[a]Days after tagging

Table 3. Fluorescence characteristics of developing cotton leaves

Leaf age[a]	F_o[b]	F_m[b]	$T_{1/2}$ (ms)	F_v/F_m
3 days	436	1963	34	0.777
8 days	414	2000	52	0.792
11 days	213	1990	110	0.893
21 days	163	1223	159	0.858
LSD 0.005	36	183	10	0.033

Data are the average of six samples
[a]Days after tagging
[b]Values are relative fluorescent units

Immunocytochemistry

To monitor the concentrations and distribution of chloroplast proteins, we probed tissue sections of the five collection dates with twelve antisera and quantified the gold particles per μm^2 of chloroplast. We also factored in the differences in total chloroplast area, although it is clear that using any one parameter in a system in which the structures are changing during development is problematic, because many parameters are changing. Because none of the antisera specifically label the starch grains, we deleted the volume occupied by starch in our calculations. This also eliminated any potential effects of amount of sunlight to which the sample was exposed on a given day or source-to-sink differences, which might cause starch levels to fluctuate. By deleting the starch quantities we also eliminated the variability of this immunologically-void tissue from our calculations of labelling density.

Several different patterns are observed in the im-

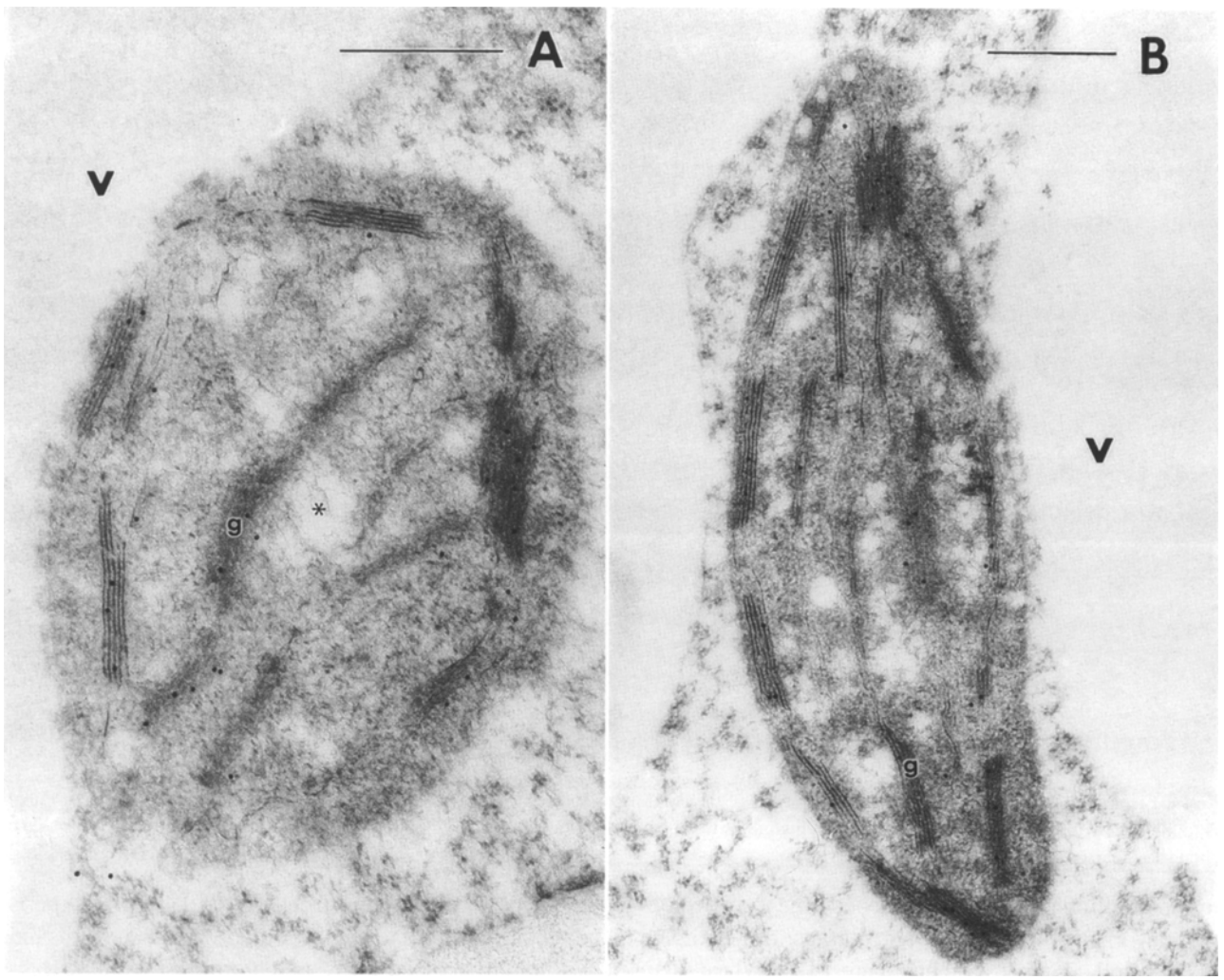

Fig. 3. Electron microscopic immunogold localizations of the PS II light-harvesting complex (LHC) in day-0 (**A**) and day-3 (**B**) cotton leaves. In both stages, LHC labelling is restricted to the tightly appressed thylakoid membranes typical of these young chloroplasts (Fig. 1). *g* Granum; *v* vacuole; asterisk, area of stroma with chloroplast DNA; bars: 0.5 μm

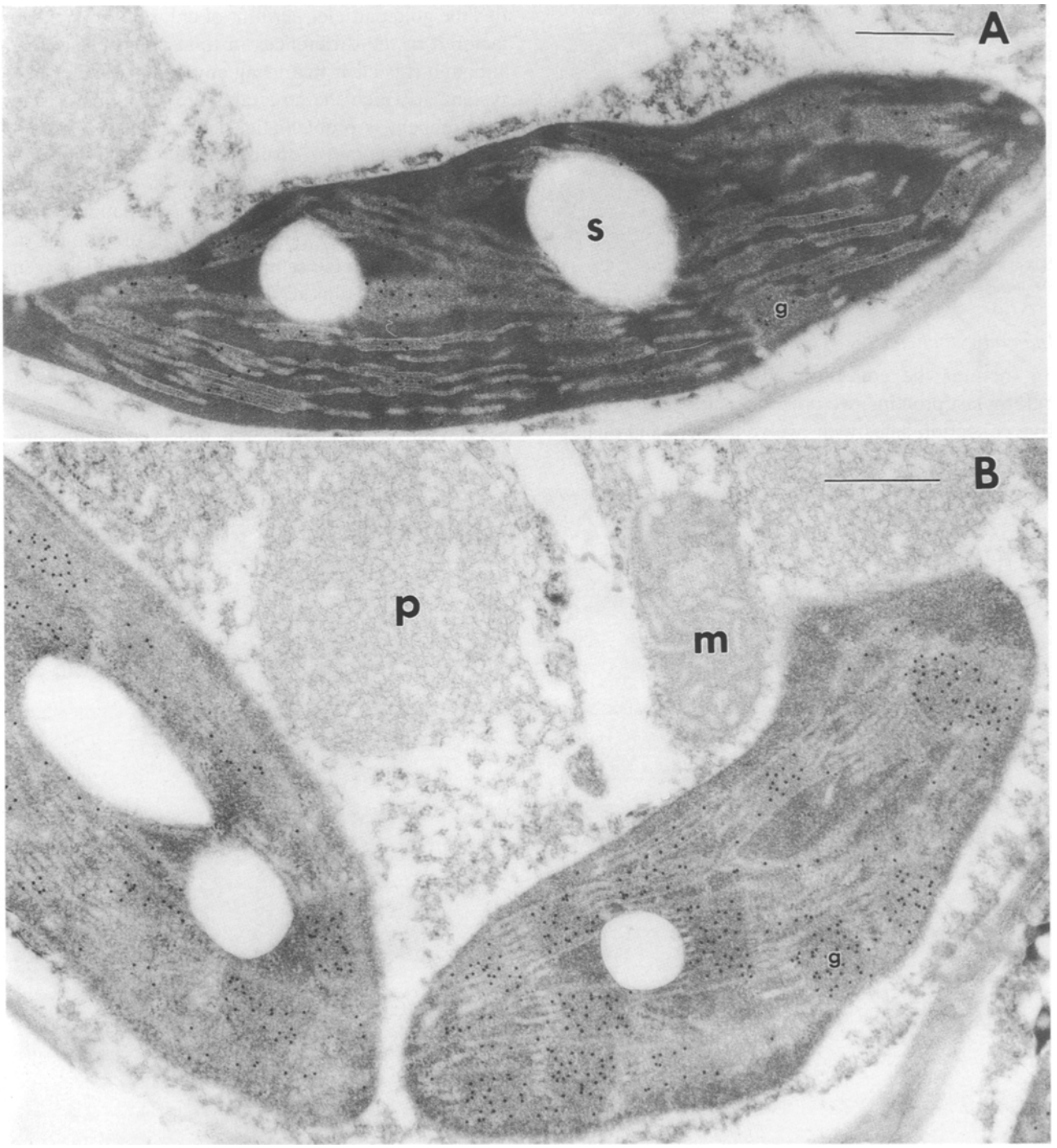

Fig. 4. Electron microscopic immunogold localizations of the PS II light-harvesting complex in day-11 (**A**) and day-21 (**B**) chloroplasts. Label is found exclusively in the grana stacks (*g*), although the stroma lamellae are well developed in these plastids. *s* Starch; *p* peroxisome; *m* mitochondrion; bars: 0.5 μm

munolabelling experiments. We describe the developmental changes of the light-harvesting complex (LHC) and ribulose bisphosphate carboxylase (RuBisCo), the principal thylakoid and stromal proteins, respectively, and the peroxisomal protein glycolate oxidase, an enzyme in the photorespiratory pathway as a comparison. Data from all the labelling experiments are summarized in Tables 4 and 5, with and without the differences in chloroplast volume factored into these numbers.

As one might expect with the low ratio of chl *a* to *b* and the great percentage of stacked membranes pre-

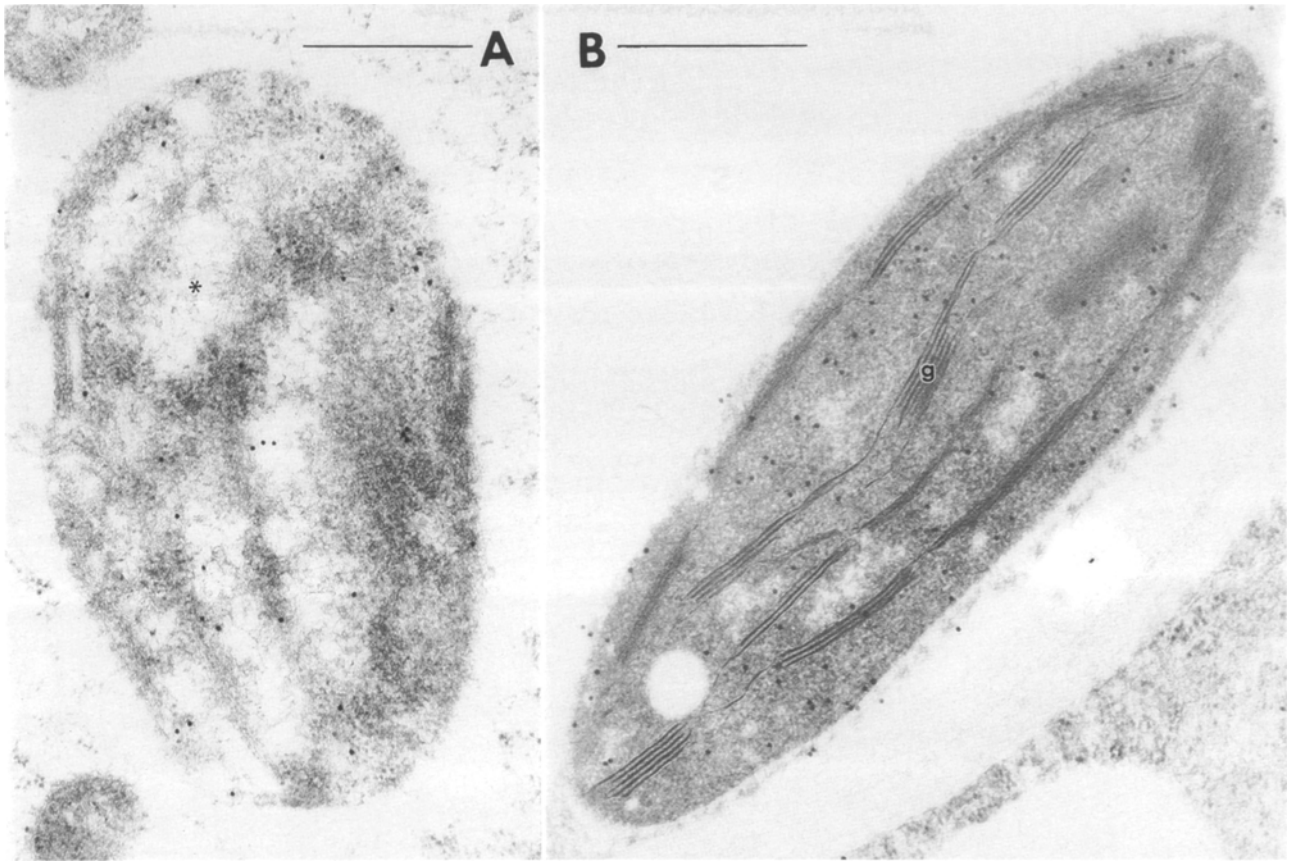

Fig. 5. Electron microscopic immunogold localizations of RuBisCo in day-0 (**A**) and day-3 (**B**) chloroplasts. Labelling is found throughout the stroma, with the exception of the clear areas containing chloroplast DNA nucleoids (asterisk), nor is labelling found on any of the thylakoid membranes. *g* Granum; bars: 0.5 μm

sent in the day-0 chloroplasts (Fig. 1 A), the labelling of the membranes with LHC antiserum is intense (Fig. 3 A), with densities based upon chloroplast area at the highest concentration of any of the collection dates. As the chloroplast increases in size in the 3-, 8- and 11-day samples (Figs. 3 B and 4 A), the LHC labelling increases on a chloroplast basis but, because these plastids are larger, the density of the labelling is less. Finally, in the day-21 samples, in which the grana stacks again increase in size, the density of LHC labelling once again increases (Fig. 4 B and Table 4). In all of these chloroplasts, virtually all of the label is found in the stacked areas of membrane and this discrimination is especially obvious in the day-21 chloroplasts in which the demarcations between stacked and unstacked thylakoids are especially pronounced. When one factors in the volume differences between the developing chloroplasts along with the densities of LHC, it appears that there are three phases of LHC accumulation in the leaves of cotton: an early accumulation in the super-stacked 0-day chloroplast, a linear phase during the increase in size of the chloroplast, and a further accumulation as grana stacks increase in size in the completely expanded leaf at day 21 (Table 4).

The labelling of the stromal protein RuBisCo (or more precisely the large subunit that is recognized by the antiserum) is found throughout the stroma with the exception of starch grains and fibrillar areas of chloroplast DNA. Strong labelling is observed even in the 0-day chloroplasts (Fig. 5 A), and are even greater in day 3 (Fig. 5 B). In the day-11 chloroplasts, the density of labelling is lower, due to an increase in volume of the plastid (Fig. 6 A). Only in the 21-day chloroplasts is the labelling density greater than in the 3-day chloroplasts (Fig. 6 B). Thus, these data on RuBisCo labelling density show similar but not identical developmental changes to that obtained on LHC accumulation. In the day-3 to -11 chloroplasts, the increase in plastid volumes outstrips the increase in RuBisCo labelling, only achieving the same density in the fully mature chloroplasts.

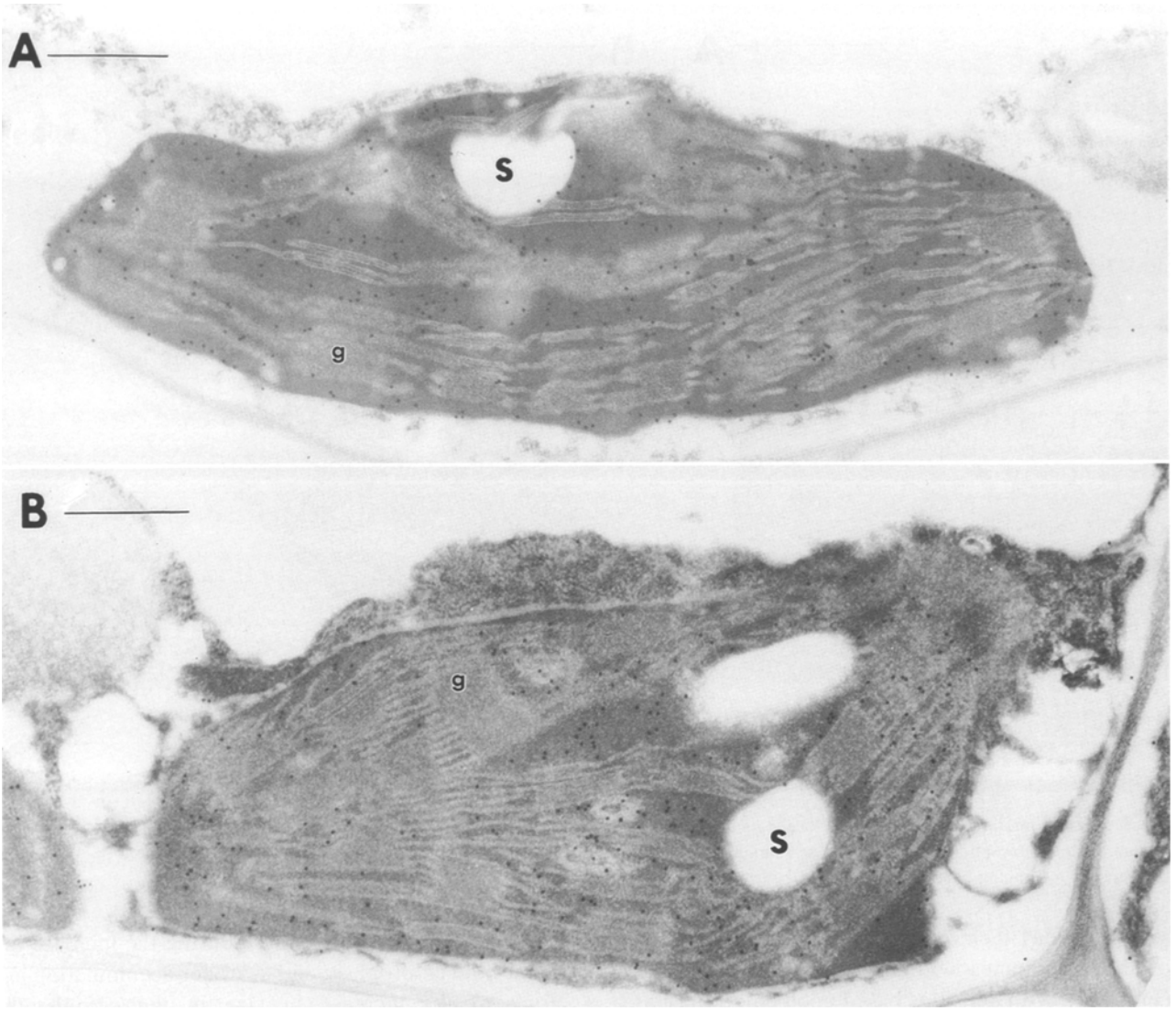

Fig. 6. Electron microscopic immunogold localizations of RuBisCo in day-11 (**A**) and day-21 (**B**) cotton chloroplasts. As in the earlier stages shown in Fig. 5, the labelling is restricted to the stroma and is essentially absent from the thylakoids and starch (*s*). *g* Granum; bars: 0.5 μm

The peroxisomal protein glycolate oxidase, the first enzyme in the photorespiratory pathway (Huang et al. 1982, Vaughn 1989), exhibits a dramatic increase in both the density of immunogold and even more so when changes in peroxisome volume are calculated at each collection date, with especially large increases at 11 and 21 days (Fig. 7).

Several other proteins (cytochrome *f*, plastocyanin, phosphoribulokinase, oxygen-evolving complex) reveal similar trends to the major thylakoid and stromal proteins, a strong labelling in either the 0- or 3-day chloroplast, more or less equivalent labelling density at the intermediate stages where chloroplast volume increases rapidly, with an increase in labelling density in the mature chloroplasts (Tables 4 and 5). However, as for LHC and RuBisCo, the volume of the chloroplast also changes dramatically during these stages, so that the total amount of labelling per chloroplast and per cell does increase, although there are no major changes in labelling density. P700 and CF1 increased only when the stroma lamellae were well developed, at 11 and 21 days, consistent with the localization of these proteins primarily in the unappressed regions of the thylakoids (Lax and Vaughn 1991). Although RuBisCo labelling density is greatest at the early stages (above), RuBisCo activase and carbonic anhydrase, which would be critical for the activity of RuBisCo, are present at the highest

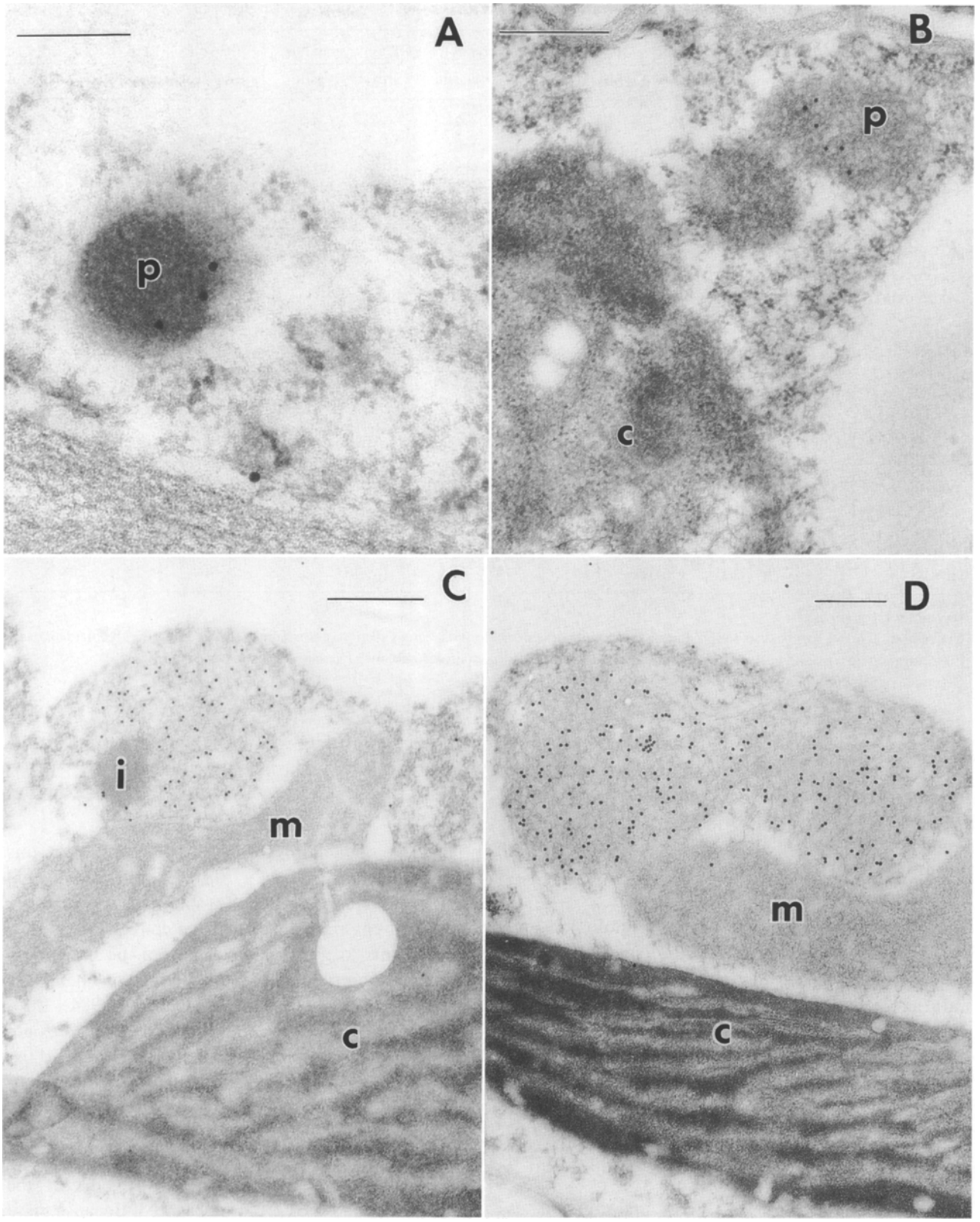

Fig. 7. Electron microscopic immunogold localizations of the photorespiratory enzyme glycolate oxidase in day-0 (**A**), -3 (**B**), -11 (**C**), and -21 (**D**) peroxisomes (*p*). In the day-0 sample, the peroxisomes are small and the amount of labelling is relatively low. Both the size of the peroxisome and the intensity of the labelling increase during the development of the cotton leaf. *i* Inclusion; *c* chloroplast; *m* mitochondrion; bars: 0.5 μm

Table 4. Concentrations of chloroplast thylakoid proteins at various leaf ages during early cotton leaf development

Leaf age[a]	LHC		OEC		Cytochrome *f*		Plastocyanin		P700		CF1	
	area[b]	plastid[c]	area	plastid	area	plastid	area	plastid	area	plastid	area	plastid
0 days	39	53	23	32	2	3	3	4	5	7	4	6
3 days	19	57	20	58	2	6	4	11	9	26	6	16
11 days	20	336	24	413	11	184	10	161	13	219	17	297
21 days	28	751	28	760	10	281	12	327	22	609	19	510
LSD 0.05	7	83	6	117	1	27	2	28	4	117	4	103

[a]Days after tagging
[b]Concentration on an area basis is for gold particles per unit area in a chloroplast cross section
[c]Concentration on a plastid basis is for gold particles per chloroplast

Table 5. Concentrations of chloroplast stroma and peroxisomal proteins at various leaf ages during cotton leaf development

Leaf age[a]	RuBisCo		RuBisCo activase		GroEl		Carbonic anhydrase		Phospho-ribulokinase		Glycolate oxidase[d]	
	area[b]	plastid[c]	area	plastid	area	plastid	area	plastid	area	plastid	area	peroxisome
0 days	29	41	1	2	17	24	1	1	1	1	1	0.1
3 days	68	197	2	4	11	32	2	6	1	4	5	3
11 days	27	436	20	346	7	118	12	202	8	132	27	70
21 days	85	2277	18	516	2	53	11	304	13	358	80	647
LSD 0.05	24	396	3	130	3	47	1	46	3	61	15	208

[a]Days after tagging
[b]Concentration on an area basis is for gold particles per unit area in a chloroplast cross section (peroxisome cross section, for glycolate oxidase)
[c]Concentration on a plastid basis is for gold particles per chloroplast (per peroxisome, for glycolate oxidase)
[d]Glycolate oxidase is a peroxisomal enzyme

concentrations at the latter stages (Table 5). Only groEl, the chaperonin, has a high density of labelling in the 0-day chloroplasts, with no great increase in labelling, despite the dramatic increase in chloroplast volume (Table 5).

Discussion

This study documents some of the physiological and structural changes that occur during the expansion of the field-grown cotton leaf from a completely unfurled leaf to a totally mature leaf, 21 days later in development. To our knowledge, this is the first time that plants grown under natural conditions of light and plant growth were monitored physiologically, structurally, and immunochemically, although other studies of cotton plants have attempted to monitor some of these parameters (e.g., Bondada et al. 1994, Constable and Rawson 1980, Pettigrew et al. 1993, Smith and Longstreth 1994). This investigation, as well as another study (unpubl.) that, because of the sample size limitations for biochemical and physiological measurements, relies only on microscopic protocols, have allowed us to learn much about the ontogeny of the chloroplast in cotton.

Chloroplast development

The development of chloroplasts described in this study presents quite a different developmental sequence than that obtained from plants grown under low-light environments, from the conversion of etiolated plants to green plants, or from the base-to-tip sequence observed in plants grown from intercalary meristems (Whatley 1974, 1978; Leech 1984). Etiolated plastids with prominent prolamellar bodies are not observed in any cell, including meristematic tissue and leaves younger than the youngest stage monitored in this study (unpubl. obs.). Likewise, single, unstacked lamellae are not found in early developmental stages, including developing plastids in the meristem. Rather, small grana, with most of the lamellae in stacks of 2–3 are observed. Because these lamellae are strongly labelled with antisera to the LHC and OEC of PS II, these represent true grana arrangements, not "pseudo-grana" as argued by some

(Valanne et al. 1981, Baker et al. 1975). These small grana stacks allow the maximum kinds of thylakoid environment, while utilizing the minimum amount of membrane. Within the small stack, the top and bottom of the stack are unappressed whereas the interior is appressed membrane. The relatively small area of the thylakoid available for proteins primarily found in unappressed membrane, such as the P700 chl *a* protein and CF1, may be limiting to electron flow in these thylakoids, perhaps explaining why more developed chloroplasts do not contain this arrangement of thylakoids. Alternately, these super-stacked membrane configurations may be a way of accommodating all the LHC that is being synthesized despite the lack of membrane and other components of the electron transport system. Numerous authors have noted that the thylakoids of young chloroplasts have a much greater electron opacity than do those from older chloroplasts (compare Fig. 1 A to Fig. 2 B) and assign a functional significance to this observation. However, polyphenol oxidase activity is highest in these young chloroplasts and the reactivity of this enzyme with endogenous phenols causes the thylakoids to appear very electron opaque (Vaughn et al. 1990). Thus, the electron opacity difference could be due to to polyphenol oxidase activity rather than an intrinsic difference in the membranes. As the leaf expands, the thylakoid ultrastructure changes in several ways: a decrease in electron opacity, an increase in the number of thylakoids per granum, and an increase in stroma lamellae. This latter change is accompanied with the appearance of the P700 chl *a* protein and CF1, proteins which are present in greater quantities in the unappressed lamellae. These data also indicate that the accumulation of proteins associated with PS II precedes those of PS I, a result which is contrary to those studies utilizing etiolated plants. These studies also indicate that there is a change in photosynthetic-unit size during the ontogeny of the chloroplast, with a relative large unit in the early stages (large amounts of LHC relative to small amounts of reaction center components such as OEC and P700 chl *a* protein) and an increasingly smaller one as the reaction center components and more reaction centers are added to the complex.

We believe that much of the difference we observe is from the way in which the plants were grown for this study in comparison with the way in which others' plants were grown. Indeed, in two of the studies that utilized plants grown under natural conditions of illumination (Ludlow 1991, Valanne et al. 1981), the results, as far as they were able to proceed, mirror our results. Even studies involving lettuce chloroplast development under relatively dim lighting (Bourdu et al. 1975) or in the slow-greening leaves of cacao (Baker et al. 1975) reveal a pattern of chloroplast ontogenesis identical to that found in our investigations. The developing chloroplasts in these studies contain thylakoids arranged in small grana at early stages and have an enrichment in chl *b*, that goes along with this preponderance of stacked lamellae. Even some of the biochemical evidence generated from intercalary meristems of monocots is supportive of the pattern of chloroplast development described herein. Mayfield and Taylor (1984) reported a strong labelling of Western blots with anti-LHC and a low ratio of chl *a* to *b* in very young leaves and a later appearance of the P700 chl *a* protein and CF1. These data give further support to our supposition that plants grown under suboptimal conditions for growth may not be an accurate reflection of normal developmental processes. Clearly, plants grown for many days in the dark before being exposed to the light or plants grown under dimly lighted conditions (many of these studies were attempted in light at 1/10 to 1/20 of that supplied by sunlight) are not the natural conditions for plant growth. Considering the difference in whole-leaf anatomy induced by low light levels (e.g., Smith and Longstreth 1994), it is not surprising that differences are noted in the patterns of chloroplast development as well.

Associations of chloroplasts with other organelles

Other cellular changes are also associated with the changes in the chloroplast and leaf development, besides those that occur within the chloroplast. At early stages of development, the plastids are intimately associated with the mitochondria, which has been ascribed to a metabolic dependence of the plastid on the mitochondria at this stage of development (Wellburn 1984). As the chloroplast develops, the vacuole expands to a great extent, with all of the organelles pushed to a thin rim of cytoplasm at the edge of the cell, forcing associations between the organelles. However, there is a consistent association between peroxisomes, mitochondria, and chloroplasts that appears to be more than circumstantial, and is probably related to the requirement of these three organelles in the photorespiratory cycle (Huang et al. 1982). The increase in both peroxisome volume and the labelling of this organelle with glycolate oxidase

antiserum give some indication of the increase in the importance of this pathway as the leaf expands.

Appearance and distribution of proteins in the chloroplast

The data presented in this report on the localization of chloroplast proteins are consistent with previous reports on the localization of these proteins (discussion in Lax and Vaughn 1991). Stromal proteins were localized exclusively to the stroma and thylakoid proteins exclusively to the thylakoids. These data indicate that the proteins are not artifactually redistributed during processing for microscopy.

The appearance of the thylakoid proteins and their relative abundance were different from what one might have expected based upon the previous studies of chloroplast development in etiolated or low-light-grown plants (Wrischer 1988, 1989). That is, one would have expected an early enrichment in PS-I-related proteins and the later appearance of PS-II-related proteins. The strong labelling of the small grana in the day-0 and day-3 thylakoids with antisera to the LHC and OEC of PS II are indicative of an enrichment in this complex in the earliest stage of chloroplast development. However, these are exactly what one would expect based upon the high degree of thylakoid appression observed in these chloroplasts and the enrichment in chl *b*. Despite the presence of these proteins in the chloroplast, the fluorescence induction kinetics indicates that even the day-3 chloroplasts are not maximally functional. At an intermediate stage of development (up until day 11), there is a tremendous increase in the size of the chloroplast and the elaboration of the thylakoid system and it is only at the last stages of chloroplast development that the density of LHC labelling again approaches the density associated with the early developmental stages. The P700 chl *a* protein- and CF1 antibody-labelling densities increase as the chloroplast develops and as the amount of unappressed membrane of the stroma lamellae increases. Again, these findings are contrary to what has been found in etiolated and low-light-grown plants. Although it was not the intention of this paper to compare the distribution of thylakoid proteins, it was surprising that the labelling of the LHC is almost exclusively associated with the grana stacks, even in the fully expanded leaves. In state-1-to-state-2 transitions, LHC is phosphorylated and there is a net movement of LHC from the grana to the stroma lamellae, allowing spillover of energy to PS I (Baker et al. 1983). Even in the fully expanded leaf, sampled at a time in which the conditions for such a change in state would be observed, virtually all of the label is observed over the grana. It is possible that the speed of the fixative reaching the chloroplast is slow enough to see some reversal of the process, which may explain the lack of any significant stroma lamellae labelling.

The stromal proteins, in general, seem to show the trend of increased labelling as the chloroplast develops. Of the stromal proteins, the most confusing localization in terms of labelling densities is RuBisCo. There is a relatively strong RuBisCo labelling in early stages, although there is still a very strong increase in labelling, especially when one factors in the increase in the size of the chloroplasts. Our antibody only recognizes the large subunit of RuBisCo so that the early strong labelling of these chloroplasts could be due, at least in part, to LSU without SSU. Indeed, the relative labelling of groEl, the chaperonin involved in complexing free LSU, is very high in day-0 and day-3 chloroplasts and progressively decreases per μm^2 of chloroplast. Similarly, the levels of RuBisCo activase, carbonic anhydrase, and phosphoribulokinase, enzymes responsible for the activation and supplying substrate for RuBisCo, increase as the leaf expands. Thus, the presence of RuBisCo labelling in the day-0 and -3 chloroplasts is most likely due to RuBisCo LSU that is present but either not in the complex with SSU, or fully activated. This same analysis could be made for all the immunological investigations described herein as the antibodies recognize the protein whether or not it is functional. All that should be said of the localization is that the protein is present and ascribe a labelling density that is reflective of its relative concentration.

Microscopic and physiological approaches to chloroplast development

The use of electron microscopy has been critical in unravelling the process of chloroplast development (Whatley 1974, 1978; Leech 1984) but has been relatively under-utilized as a tool since the advent of immunocytochemical protocols. These immunological techniques have allowed for localization and quantification of proteins for which standard biochemical protocols could not be utilized readily. Coupling these techniques with those physiological protocols utilizing fluorometry and very-small-chamber

gas exchange analysis should allow one to determine many facets of chloroplast development and with a degree of precision previously unobtainable.

In summary, we have found that cotton chloroplast development, under conditions of natural light and growth, are different from that described from plants grown under low-light regimes or released from continuous darkness. The development of appressed membranes enriched in PS-II-related proteins occurs prior to the development of extensive unappressed lamellae, with proteins associated with PS I appearing (or increasing) coincidently with the appearance of these unappressed lamellae. Although we have only examined cotton in this report, what other studies have been conducted in a similar matter with other species appear to be consistent with the pattern of development described herein for cotton.

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